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Original Article

Characterization of follistatin isoforms in early *Xenopus* embryogenesis

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ABSTRACT Follistatin is expressed in Spemann's organizer in the *Xenopus* gastrula and mimics the activity of the organizer, inducing a neural fate directly in the ectoderm. We have previously shown that follistatin inhibits BMP activity through a direct interaction. In this study, we have characterized the localization and function of two follistatin isoforms to examine the functional differences between them. One notable difference, previously described, is that the shorter form (xFSS or xFS319) but not the C-terminally extended long form (xFSL) associates with cell-surface matrices. Here, we show that the spatial-temporal expression pattern of xFSL and xFSS is indistinguishable. Interestingly, however, xFSS was found to have a more potent inhibitory activity against BMP-4 than xFSL. Furthermore, using a surface plasmon resonance biosensor, xFSS was shown to have a higher binding capacity for BMP subtypes. The diffusion rates of xFSS and xFSL ectopically expressed in *Xenopus* embryos were similar. Taken together, our results suggest that the difference in BMP-inhibiting activity of the two follistatin isoforms is mainly attributable to a difference in their BMP binding properties rather than to their diffusion rates.

KEY WORDS: *follistatin, Xenopus, organizer, BMP.*

Introduction

The development of the vertebrate nervous system begins with the induction of the neural fate in the dorsal ectoderm by Spemann's organizer. Recent studies of neural induction in *Xenopus* have revealed that Spemann's organizer secretes molecules so-called organizer factors, which include noggin (Smith and Harland, 1992), chordin (Sasai *et al*., 1994), and follistatin (Hemmati-Brivanlou *et al*., 1994), all of which directly neuralize the ectoderm, which would otherwise give rise to epidermis. *Xenopus* epidermis is known to be induced in the ectoderm by a polypeptide growth factor belonging to the TGF-β superfamily, bone morphogenetic protein (BMP), which is localized in the ventral side of the ectoderm and mesoderm of early gastrula embryos. In addition to its ability to ventralize mesoderm, BMP has a potent anti-neuralizing activity when overexpressed, inducing an epidermal fate in the ectoderm (Hemmati-Brivanlou and Melton, 1997; Suzuki *et al*., 1995; Wilson and Hemmati-Brivanlou, 1995). This anti-neuralizing activity is similar to the role of *Drosophila* dpp (Holley *et al*., 1995; Holley *et al*., 1996), a counterpart of vertebrate BMP; in loss-of-function mutants of dpp, the neuroectoderm, which is normally formed ventrolaterally, expands to the dorsalmost part of the *Drosophila* embryo.

Furthermore, recent studies also revealed the molecular nature of Spemann's organizer: all the organizer factors have been shown to interact directly with BMP and prevent BMP from activating its receptor (Fainsod *et al*., 1997; Iemura *et al*., 1998; Piccolo *et al*., 1996; Zimmerman *et al*., 1996), suggesting that they induce the neural fate by inhibiting the anti-neuralizing (epidermis-inducing) activity of BMP. In addition, a head-inducing organizer factor, Cerberus (Bouwmeester *et al*., 1996) has also been found to bind and inhibit BMP-2/4. These studies have resulted in a revision of the previous interpretation of Spemann's experiment in which neural fate was thought to be actively induced by the organizer, and led to a new model in which the

Abbreviations used in this paper: BMP, bone morphogenetic protein : RT-PCR, reverse transcription polymerase chain reaction; HA, hemagglutinin.

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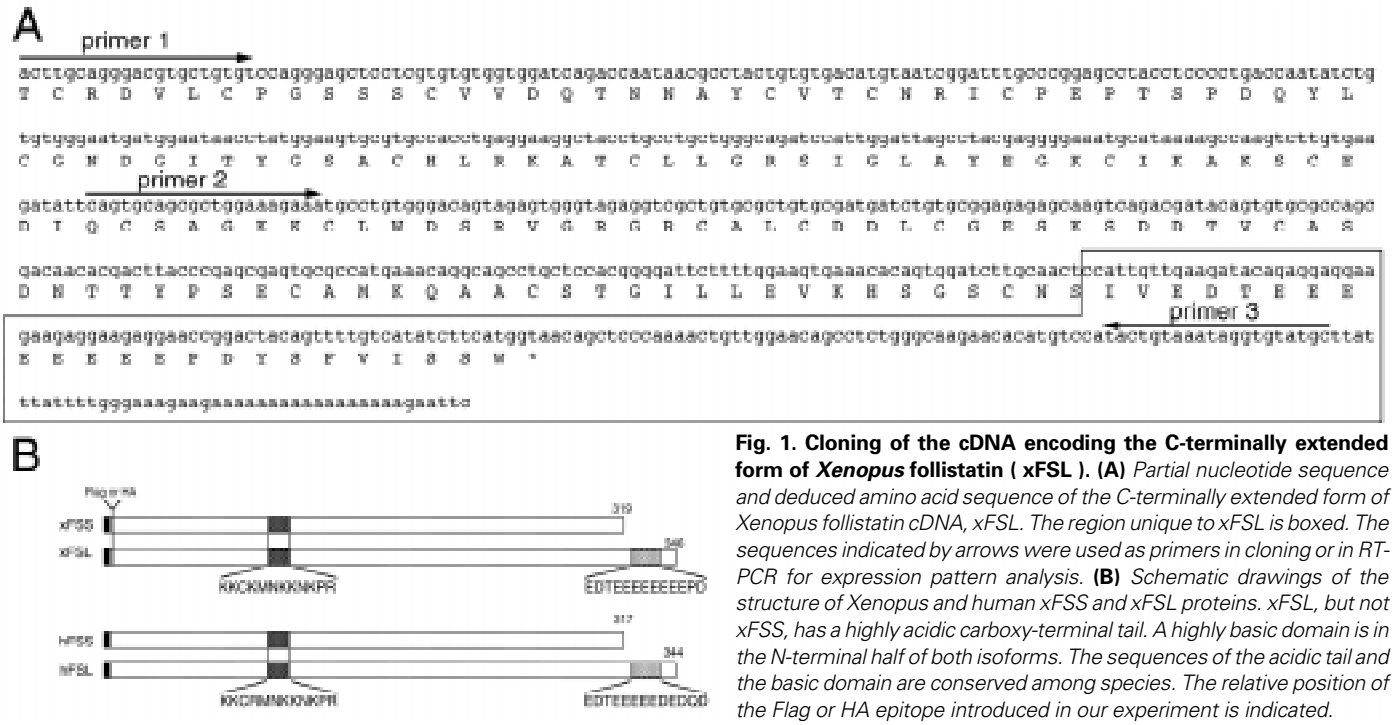

Fig. 1. Cloning of the cDNA encoding the C-terminally extended form of *Xenopus* follistatin (xFSL). (A) *Partial nucleotide sequence and deduced amino acid sequence of the C-terminally extended form of Xenopus follistatin cDNA, xFSL. The region unique to xFSL is boxed. The sequences indicated by arrows were used as primers in cloning or in RT-PCR for expression pattern analysis.* **(B)** *Schematic drawings of the structure of Xenopus and human xFSS and xFSL proteins. xFSL, but not xFSS, has a highly acidic carboxy-terminal tail. A highly basic domain is in the N-terminal half of both isoforms. The sequences of the acidic tail and the basic domain are conserved among species. The relative position of the Flag or HA epitope introduced in our experiment is indicated.*

neural fate is believed to be a ground or default state, so that the only cells to adopt the neural fate are those that are not induced to form epidermis by the influence of BMP.

The *in vivo* roles of BMP and its antagonist, chordin, in vertebrate embryogenesis, has recently been demonstrated by zebrafish genetics. A dorsalized mutant, *swirl*, which displays phenotypes of expanded dorsal mesoderm and neural tissues, was found to result from mutations of the *zbmp-2* (*zebrafish BMP-2*) gene (Kishimoto *et al.*, 1997; Miller-Bertoglio *et al.*, 1997), indicating that zebrafish BMP-2 (Nikaido *et al.*, 1997) is essential for ventralization of the embryo *in vivo*. Furthermore, a ventralized mutant, *dino* (*chordino*), whose neuroectoderm and spinal cord interneurons are significantly reduced, was found to be a loss-of-function mutation of zebrafish *chordin*, further supporting the idea that the antagonism between BMP and chordin is essential for neural induction and patterning *in vivo*.

BMP-4 in *Xenopus* and BMP-2 in zebrafish are known to be uniformly distributed before gastrulation, and then to become restricted to the ventral ectoderm by the influence of the organizer and shield, respectively. The restriction in expression is presumably due to the inhibition of the BMP gene's self-activated expression loop by organizer factors. This observation suggests that a gradient of BMP activity is formed by the negative regulation of organizer factors, as well as by the direct but restricted diffusion of BMP proteins (Nikaido *et al.*, 1999). The gradient of BMP activity from the ventral to dorsal ectoderm may be quite important for the patterning of the neuroectoderm, as shown previously for the patterned target gene expression regulated by different concentrations of activin (Green *et al.*, 1992). Although more is being learned about the antagonistic interaction between BMP and organizer factors, it is not fully understood how distribution of the organizer factors to the ventral ectoderm is controlled; i.e., does it depend on free diffusion from the organizer, or is it restricted to the organizer resulting in a steep gradient?

Follistatin was originally purified as a protein with an activity opposite to that of activin; that is, follistatin inhibited FSH secretion from pituitary cells (Ueno *et al.*, 1987). Later, follistatin was found to be an activin-binding protein (Nakamura *et al.*, 1990). However, recent studies have demonstrated that it also binds to and inhibits BMPs, although it binds to BMP with a lower affinity than to activin (Fainsod *et al.*, 1997; Iemura *et al.*, 1998). There are two isoforms of follistatin that are generated by an alternative splicing event from a single gene (Shimasaki *et al.*, 1988; Tashiro *et al.*, 1991). Interestingly, it was reported that the shorter form has a tendency to be trapped on the cell surface via a region of basic amino acids (Sugino *et al.*, 1993). These findings led us to examine first, whether the two isoforms inhibit BMP activity with different potencies, and second, whether the binding of follistatin to the cell surface restricts its diffusion from the organizer.

Results

Cloning of the long form of *follistatin* from *Xenopus*

Two subtypes of follistatin have been identified in human: follistatin 288 (FS288) and a C-terminally extended form of FS288 (FS315) (Shimasaki *et al.*, 1988). These two isoforms, tentatively designated as FSS and FSL, respectively, are generated by the alternative splicing of a single *follistatin* gene (Shimasaki *et al.*, 1988). The shorter FSS tends to bind to the membrane surface, probably to extracellular matrices harboring negatively charged carbohydrate chains, such as heparan sulfate, via a region of basic amino acids in the N-terminal half of the molecule (Fig. 1B)(Sugino *et al.*, 1993). The extra C-terminal sequence in FSL is rich in acidic amino acids (Fig. 1B) (Shimasaki *et al.*, 1988). This highly acidic domain is

believed to prevent the basic region from interacting with heparan sulfate, because FSL has little binding affinity for the cell surface compared to FSS (Sugino *et al.*, 1993). It is also speculated that the acidic domain interacts with the basic domain, directly masking the basic domain. In *Xenopus*, although the presence of a long form corresponding to human FSL was previously reported (Tashiro *et al.*, 1991), little is known about the function of *Xenopus* FSL (xFSL). In contrast, a cDNA for *Xenopus* FS319 (xFSS), which corresponds to human FSS has been cloned and found to be an organizer factor that can directly induce a neural fate in the presumptive ectoderm (Hemmati-Brivanlou *et al.*, 1994). In this study, our first goal was to characterize the localization and biological function of xFSL and to compare these properties with those of xFSS.

According to the previous reports (Shimasaki *et al.*, 1988), oligonucleotide primers corresponding to a conserved sequence close to the C-terminus (Fig. 1A, primer 1) and poly(A), were synthesized and polymerase chain reaction (PCR) was performed. Amplified DNA fragments were subcloned into BlueScript, and colonies were randomly picked for sequencing. One of the clones was found to encode a sequence identical to the previously reported C-terminally extended region of xFSL (Fig. 1). This sequence was subsequently ligated to a fragment of xFSS (FS319) that had been digested with Sac I, to generate a full-length cDNA of xFSL. This xFSL cDNA was then used for functional studies.

Localization of *xFSS* and *xFSL* transcripts during embryogenesis.

xFSS was previously shown to be expressed in a few cells in the Spemann's organizer at the beginning of gastrulation, consistent with its proposed function as an organizer factor (Hemmati-Brivanlou *et al.*, 1994). To examine the developmental expression pattern of *xFSL*, reverse transcription PCR (RT-PCR) was performed using specific primer sets for *xFSS* and *xFSL* (Fig. 1A, primer 2 and 3). Both transcripts were first detectable at stage 9, around the onset of the midblastula transition, suggesting that *FSS* and *FSL* are not maternally but zygotically expressed (Fig. 2A). We next examined the localization of each transcript. Because it was not possible to prepare RNA probes specific to each subtype with sufficient length for *in situ* hybridization, we detected the transcripts by RT-PCR using RNAs prepared from different regions of embryos dissected at stage 10.5. As previously reported, the *xFSS* transcript was found to be abundant in the dorsal marginal zone, forming a gradient from the dorsal to the ventral side of the embryo (Fig. 2B, upper panel). The expression pattern of *xFSL* was indistinguishable from that of *xFSS* (Fig. 2B, lower panel). This suggests that xFSL may also function as an organizer factor.

Functional difference between xFSS and xFSL

Although follistatin was originally identified as an activin-binding protein (Nakamura *et al.*, 1990), recent studies suggest that follistatin can directly interact with BMP to inhibit its activity (Iemura *et al.*, 1998). To examine whether xFSS and xFSL inhibit BMP activity with different potencies, we performed functional studies using *Xenopus* embryos. As previously shown, overexpression of xFSS in the ventral blastomeres, where endogenous BMP-4 is thought to act, resulted in the duplication of the dorsal axis in a dose-dependent manner, presumably by blocking the ventralizing activity of the BMP. The secondary axis was induced by the injection of 50 pg of xFSS mRNA (Fig. 3, A, C, E). In contrast, xFSL mRNA exhibited little effect on secondary axis formation, even at 100 pg (Fig. 3, B, D, F). These results, summarized in Table 1, suggest that xFSL has a weaker inhibitory activity for BMP than does xFSS. This result was further confirmed by following marker gene expression in animal cap and ventral marginal zone explants (Fig. 4A). Briefly, *N-CAM* and *XAG-1*, neural specific markers in the animal cap, and *goosecoid* and *α-actin*, dorsal mesodermal markers in the marginal zone were more efficiently induced by xFSS than by xFSL, indicating that xFSS is more potent than xFSL in inhibiting BMP activity. In fact, xFSS but not xFSL induced evident cement glands in animal cap explants (Fig. 4B). This is unlikely to be due to differences in translation efficiency because the ectopic expression of hemagglutinin (HA)-tagged xFSS and xFSL in the *Xenopus* animal cap gave a similar intensity of immunoreactivity to HA antibodies (Fig. 6B).

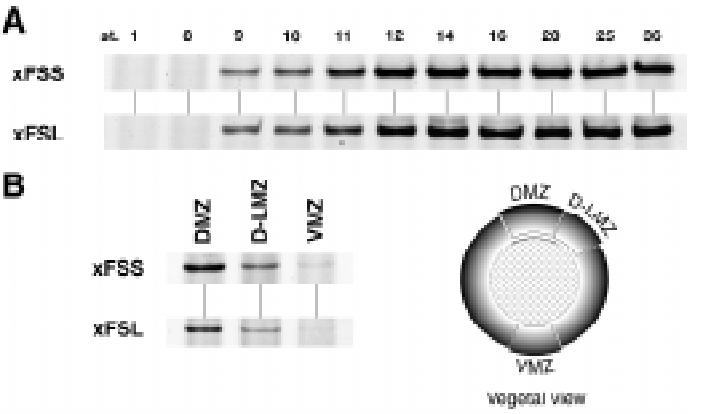

Fig. 2. Expression pattern of follistatin isoforms. (A) *Developmental expression of follistatin isoforms in Xenopus whole embryos by RT-PCR.* **(B)** *Spatial expression of follistatin isoforms in the marginal zone of Xenopus gastrula by RT-PCR. Xenopus gastrula embryos were dissected as shown diagrammatically on the right and RNAs from the same number of tissue pieces were analyzed by RT-PCR. DMZ, dorsal marginal zone; D-LMZ, dorsolateral marginal zone, VMZ, ventral marginal zone. The primers used in this analysis are indicated in Fig. 1A. The upper xFSS signal is ~600 bp and the lower xFSL signal is 350 bp.*

Difference in binding affinity of xFSS and xFSL to BMP

To examine how the difference in inhibitory activity of the two isoforms is derived, we measured the affinity of xFSS and xFSL for BMP-4 using a BIACORE surface plasmon resonance sensor. This instrument allows the detection of weak protein interactions in real time, without having to label the proteins. We have previously demonstrated the direct binding of follistatin to BMP and showed that the dissociation rate of this binding is rather fast compared with the dissociation rate of follistatin binding with activin (Iemura *et al.*, 1998). Here, we used a human recombinant protein of each isoform, hFSS (FS315) and hFSL (FS288), corresponding to xFSS and xFSL, respectively (Fig. 1), as an analyte. hFSS gave a similar binding profile to BMP-4 to that reported previously (Fig. 5). However, binding response of hFSL to BMP-4 was significantly lower than that of hFSS (Fig. 5), indicating that the affinity of BMP for hFSL is much lower than for

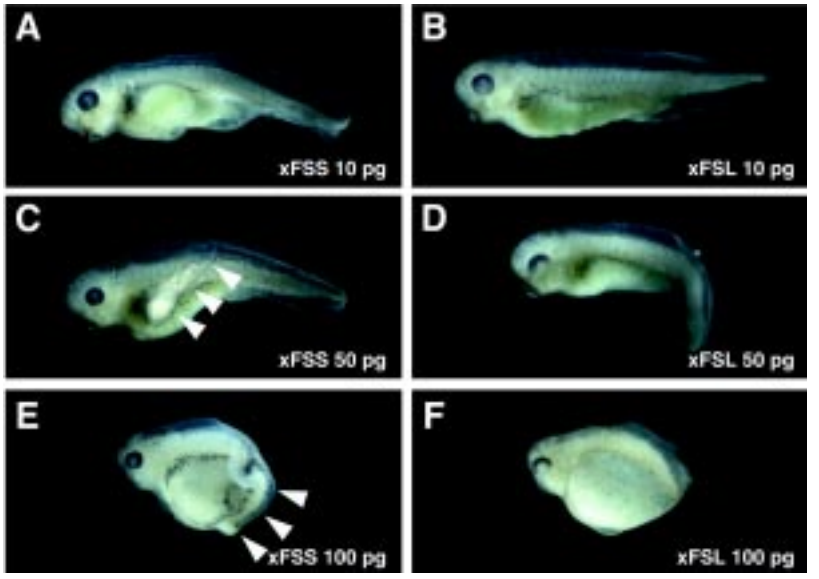

Fig. 3. Ectopic dorsal axis formation and hyperdorsalization induced by follistatin isoforms. *Phenotypes of Xenopus embryos ventrally injected with the mRNA for follistatin isoforms. Increasing amounts of mRNA for the Xenopus follistatin isoforms indicated were injected into the equatorial region of the two ventral blastomeres at the 4-cell stage. Embryos ventrally overexpressing xFSS effectively formed ectopic dorsal axes, but embryos overexpressing xFSL did so only rarely. pg, picogram.*

hFSS. Similar results were obtained about the binding response of hFSL and hFSS to BMP-2 and -7 (data not shown). This results suggest that the weaker BMP-inhibiting activity of xFSL is due to a lower BMP-binding capability.

The diffusion rate of follistatin isoforms is comparable

Porcine FSS and FSL have different cell-surface attachment properties, as demonstrated with ovarian granulosa cells (Sugino *et al.*, 1993). FSS binds to the cell surface, probably via a basic domain in the N-terminal half of the molecule; such a domain has a tendency to bind the negatively charged carbohydrate chains of cell-surface proteoglycans. Consistent with this, the extra acidic domain of FSL cancels the binding ability of FSS (Sugino *et al.*, 1993), probably because the basic domain is masked intramolecularly by the acidic domain by an electrostatic interaction. An interaction between xFSS and proteoglycans might influence its diffusion rate. Furthermore, if xFSL has a less efficient interaction with the cell surface, it might travel farther in the intercellular space than does xFSS. To examine the importance of the basic domain in restricting the range of follistatin diffusion, we tested the diffusion rates of xFSS and xFSL. Both follistatins were efficiently produced and secreted from 293T cells transiently transfected with Flag-xFSS or Flag-xFSL cDNA, and no obvious difference in the secretion rate into medium or the amount retained in the cells was observed (Fig. 6A). This was also confirmed in animal cap cells (Fig. 6B). Functional analysis of epitope-tagged FSS and xFSL in *Xenopus* embryos demonstrated that they retained not full, but significant BMP-inhibiting activity (data not shown). We next tested the diffusion rate in *Xenopus* embryos by injecting differently tagged follistatin mRNAs into the animal pole and detecting them by immunofluorescent staining. Two-cell stage embryos were coinjected with mRNAs encoding Flag-tagged xFSS and HA-tagged xFSL at the animal pole and grown to the blastula stage. Animal caps from the embryos were dissected and subjected to immunofluorescent staining. The results showed that both follistatin isoforms were located largely in the vicinity of the cells expressing each isoform and that there was no obvious difference in the localization of the two proteins (Fig. 7). A similar result was obtained in an experiment where the tags were reversed, i.e., HA-tagged xFSS and Flag-tagged xFSL (data not shown). These results suggest that neither xFSS nor xFSL may be able to travel far from the vicinity of the cells expressing them. Based on these results, we tentatively conclude that the diffusion rates of both follistatin isoforms are comparable in *Xenopus* animal cap cells.

Discussion

The actions of many biologically active substances are negatively regulated by their specific inhibitors during early embryogen-

TABLE 1

ACTIVITY OF xFSS AND xFSL TO FORM ECTOPIC AXIS AND TO HYPERDORSALIZE BY MICROINJECTION

	Survivor		Phenotype %							
mRNAs injected	%	(n)	DAI 5	DAI 6	DAI 7	DAI 8	DAI 6 S.A.	DAI 7 S.A.	Others	Av.DAI
xFSS (10 pg)	100	(34)	64	36	-	-	-	-	-	5.36
xFSS (50 pg)	100	(37)	11	-	-	-	73	16	-	6.05
xFSS (100 pg)	98	(41)	-	7	29	2	10	50	-	6.85
xFSL (10 pg)	100	(33)	91	9	-	-	-	-	-	5.09
xFSL (50 pg)	100	(36)	72	28	-	-	-	-	-	5.28
xFSL (100 pg)	97	(38)	13	63	21	-	-	-	-	6.08
uninjected	100	(40)	91	2	-	-	-	-	7	5.02

Phenotypes were estimated after 60 hours of development.
DAI, the dorsoanterior index described by Kao and Elinson (1988); sS.A., secondary dorsal axis as seen in Fig.3C,E; Others, abnormalities including dorso-posterior defect, incomplete invagination and spontaneous ventralization.

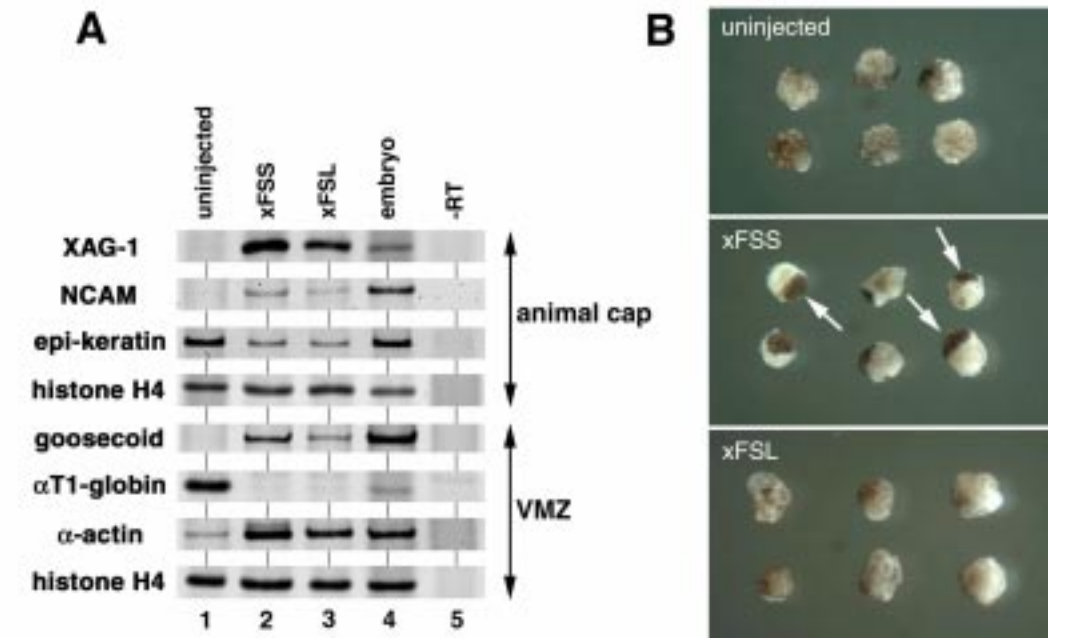

Fig. 4. xFSL has weaker neuralizing and dorsalizing activities than xFSS in embryonic explants. (A) *Expression of molecular markers in animal cap and ventral marginal explants. Embryos were injected with the follistatin mRNAs indicated at the top of each lane into the animal pole of both blastomeres at the 2-cell stage for the animal cap or into the equatorial region of the two ventral blastomeres at the 4-cell stage for the marginal zone assay. Sixty picograms of each follistatin mRNA was injected. Animal caps and ventral marginal zones were excised and explanted at the blastula and early gastrula stage, respectively, and incubated until sibling embryos reached stage 11, 25, or 36.* **(B)** *Morphological analysis of animal caps at stage 25. Animal cap explants injected as in (A) were incubated until sibling embryos reached stage 25. Cement glands were induced effectively in xFSS-injected animal caps (white arrows), but rarely in xFSL-injected ones.*

esis (Perrimon and McMahon, 1999; Thomsen, 1997). In the case of polypeptide growth factors, such inhibitors include their own precursor peptide (Miyazono *et al.*, 1988), receptor-like proteins (Leyns *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997), and even structurally unrelated binding proteins (Hsu *et al.*, 1988; Iemura *et al.*, 1998; Piccolo *et al.*, 1999; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). It is believed that both growth factors and such negative regulators contribute to the establishment of gradients or boundaries of growth factor activities (Graff, 1997; Moon *et al.*, 1997).

In this study, we compared various properties of FSS and FSL, both of which are proposed to be BMP antagonists, to see if there were functional differences between them. First, we found that FSS has more potent dorsalizing activity than FSL when its mRNA was injected into the animal pole or ventral marginal zone (Figs. 3, 4). It has recently been demonstrated that follistatin dorsalizes the embryo by inhibiting BMP activity through direct binding (Iemura *et al.*, 1998). We therefore predicted that FSS has a higher binding affinity than FSL to BMPs, which are known to have ventralizing activity. As we expected, FSS showed significantly higher binding capacity to BMP-4 and BMP-7 (Fig. 5). Based on the structural differences between FSS and FSL (Fig. 1), it is presumed that the acidic amino acid region in the C-terminus of FSL attenuates its ability to bind BMPs. The amino acid sequence EDTEEEEEDEDQD in FSL might electrostatically interact with the basic amino acid sequence KKCKMNKKNKPR in its N-terminal half, thereby changing its conformation. This intramolecular looping of the FSL polypeptide may lead to a loss of its ability to bind BMP.

A second possibility was that the two isoforms have different functional ranges as a result of their structural differences, since the acidic domain of FSL is also thought to decrease the association of follistatin's basic domain with cell surface molecules. To explore this possibility, we examined whether or not a difference in the diffusion rates of the isoforms could account for the differences in their inhibitory activity against BMPs observed *in vivo*. However, our analysis using Flag-tagged and HA-tagged follistatin isoforms suggests that neither form diffuses efficiently from its site of biosynthesis in *Xenopus* embryos, regardless of the tag used (Fig. 7). These results also indicated that the two follistatin isoforms did not have different diffusion rates. The amino acid sequence of the Flag epitope is quite acidic

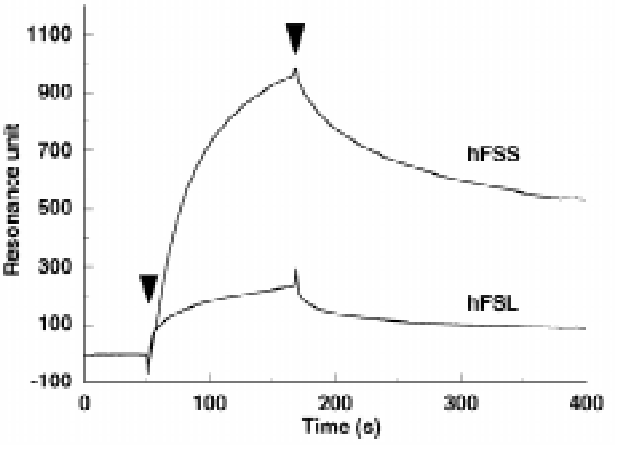

Fig. 5. Direct binding capability of follistatin isoforms to immobilized BMP-4. *BMP-4 was immobilized on the sensor chip surface (2904 RU). hFSS or hFSL, at a concentration of 5 μg/ml, was injected over the BMP-4 surface at a flow rate of 20 μl/min for 120 s at 25 °C. Arrowheads represent the initiation and termination of injections.*

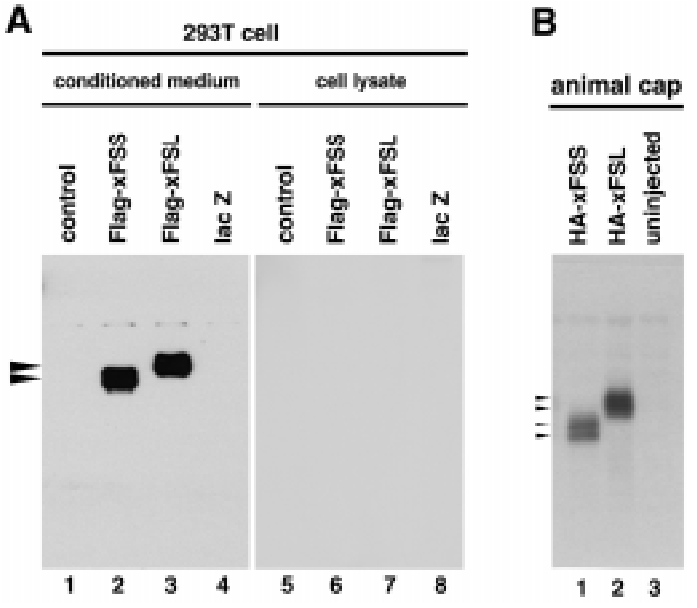

Fig. 6. Both xFSS and xFSL proteins are comparably synthesized in and secreted by mammalian cells and *Xenopus* embryos. *Western blot analysis of (A) Flag-tagged and (B) HA-tagged follistatin isoforms in (A) 293T cells and (B) the animal cap of Xenopus embryos.* **(A)** *293T cells were transiently transfected with 5 μg of Flag-xFSS (lanes 2, 6), Flag-xFSL (lanes 3, 7), or lacZ (lanes 4, 8) constructs. The conditioned medium (lanes 1-4) and the cell lysate (lanes 5-8) were analyzed using a monoclonal anti-Flag antibody.* **(B)** *Embryos were injected with 100 pg of HA-xFSS (lane 1) or HA-xFSL (lane 2) mRNA into the animal pole of both blastomeres at the 2-cell stage. The excised animal cap from each embryo was analyzed by Western blotting using polyclonal anti-HA antibodies. Immunoreactive signals are indicated by arrow heads.*

(DYKDDDDK), making the Flag-tagged fusion proteins more acidic than wild-type or HA-tagged follistatins. The observation that even the xFSL proteins fused with the Flag tag were restricted to the injection site and colocalized with HA-tagged xFSS suggests that the acidic region is not critical for determining the diffusion distance, and that intact forms of both xFSS and xFSL are restricted to the site of biosynthesis, the organizer. Together, these results suggest that the differences in the inhibitory activity of follistatin isoforms for BMPs depend solely on differences in their ability to bind BMPs. It was, however, previously shown by Sugino *et al.* (1993) that the follistatin isoforms have different binding abilities to rat granulosa cell surface, that is, FSS binds to the cell surface more efficiently than FSL, and consequently, FSS shows more potent suppressing activity in follicle-stimulating hormone release than FSL. This discrepancy may be explained by two possibilities. One is that the cell systems used in analysis are different, *Xenopus* embryo and rat granulosa cell culture. The composition of extracellular matrix of these cells may be different in binding of the follistatin isoforms. The other is that in Sugino *et al.* (1993) the difference in binding ability of follistatin isoforms to cell surface was determined as the amount of ^{125}I-labeled activin molecule binding to the cell surface follistatin, based on the result that FSS and FSL had a similar specific activity to bind activin. However, our data using BIACORE showed that hFSS was more potent in binding not only to BMP but also to activin than hFSL (data not shown).

The results in this study further suggest that follistatin distribution is such that it forms a steep gradient of anti-BMP activity that is highest in the organizer. In contrast, noggin and chordin (sog) are believed to be highly diffusible (Ashe and Levine, 1999; Dosch *et al.*, 1997). Therefore, it is possible that the overlapping action of these organizer factors with different diffusion rates creates a gradient of inhibitory activity against the BMPs, which are produced ventrally, that is highest in the organizer.

Although the organizer factors that have been identified so far, including noggin, chordin, follistatin have been found to act by a similar mechanism, the contribution of each factor to neural induction is not clear. Single gene disruption of either *noggin* or *follistatin* in the mouse and *chordino* in the zebrafish indicates that no single one of these genes has a critical role in neural induction per se. However, the possibility remains that there is redundancy and the cooperative action of several organizer factors is important to trigger neural induction. Further functional studies will be required before a complete understanding of the nature of organizer activity is achieved.

Materials and Methods

Cloning of cDNA for the long form of *Xenopus* follistatin (xFSL)
Two isoforms of human follistatin, a short form and C-terminally extended long form, are known to be made by alternative splicing in the 3' region of this gene. To isolate cDNA encoding xFSL we performed a 3' RACE experiment. An oligonucleotide corresponding to the conserved sequence, 5'-ACTTGCAGGGACGTGCTGTGT-3',
as the upstream primer and oligo dT with an EcoRI recognition site, 5'-GAGTCGACTCGAGAATTCTTTTTTTTTTTTTTTT-3', as the downstream primer were synthesized, and RT-PCR was performed using RNA extracted from *Xenopus* stage 11 embryos. DNA fragments amplified to the expected size were ligated into the cloning vector BlueScript SK+ and sequenced. The EcoRI-SacI fragment of a cDNA clone encoding the C-

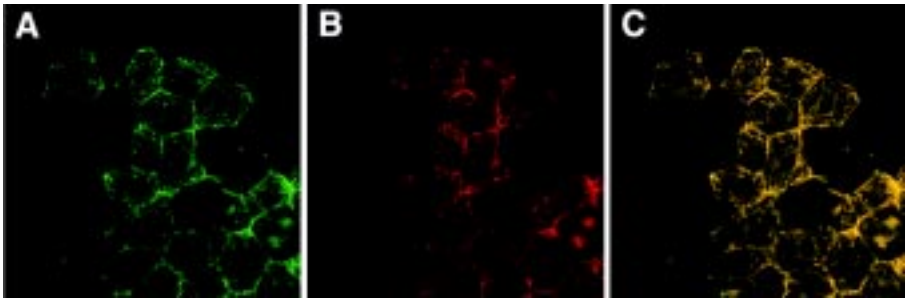

Fig. 7. Colocalization of xFSS and xFSL proteins. *100 pg of Flag-xFSS and HA-xFSL mRNA were co-injected into the animal pole of 2-cell stage embryos. At the blastula stage the animal hemisphere was dissected and analyzed for immunofluorescence.* **(A)** *xFSS protein was detected with a monoclonal anti-Flag antibody with a Cy5 conjugated anti-mouse secondary antibody (green) and* **(B)** *xFSL protein was visualized using a polyclonal anti-HA antibody with a rhodamine conjugated anti-rabbit secondary antibody (red). The expressed proteins were visualized using confocal microscopy.* **(C)** *The double immunofluorescence (yellow) demonstrates that the xFSS and xFSL proteins are mostly colocalized in the vicinity of the expressing c*ell.

terminal part of xFSL was fused with the SacI digested 5' portion of xFSS cDNA, resulting in a cDNA for full-length xFSL.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Gene expression in animal caps, marginal zone explants, and embryos was analyzed by RT-PCR. Briefly, total RNA was extracted using TRIzol reagent (GIBCO/BRL) according to the manufacturer's instructions and was subjected to the reverse transcription reaction with random primers. PCR-amplified products from the RT mixture using specific primers to each gene were detected using Fluorimage analyzer FLA2000 (FUJIFILM). The nucleotide sequences of the specific primers used in this study were as follows:

those for goosecoid, aT1-globin, and histone H4 were as described previously (Iemura *et al*., 1998),

XAG-1, upstream, 5'-CTGACTGTCCGATCAGAC-3'
and downstream, 5'-GAGTTGCTTCTCTGGCAT-3';

NCAM, upstream, 5'-GCCTGTAGAATTACAATGCTG-3'
and downstream, 5'-AGCATCTTGGCTGCTGGCATT-3';

epidermal keratin, upstream, 5'-CACCAGAACACAGAGTAC-3'
and downstream, 5'-CAACCTTCCCATCAACCA-3';

a-actin, upstream, 5'-TCCCTGTACGCTTCTGGTCGTA-3'
and downstream, 5'-TCTCAAAGTCCAAAGCCACATA-3';

follistatin, upstream, 5'-CAGTGCAGCGCTGGAAAGAA-3'
and downstream, 5'-GCATACACCTATTTACAGTA-3'.

Embryo manipulations and microinjection

Fertilization, embryo culture, and microinjection of synthetic RNAs were carried out as described previously (Yamamoto *et al*., 2000). Unfertilized eggs were collected and fertilized *in vitro*. Embryos were dejellied using 3% cysteine and washed with water several times. Capped mRNAs were synthesized *in vitro* using the mMESSAGE mMACHINE sp6 kit (Ambion). Synthesized RNAs were injected into the animal pole or marginal zone of 2- or 4-cell stage embryos, respectively, in 3% Ficoll/ 0.1x Steinberg's solution as described previously (Suzuki *et al*., 1997). For the evaluation of mesodermal markers, the ventral marginal zone was excised at stage 10. For ectodermal markers, the animal cap was dissected at stage 8.5. The explants were cultured in 0.1% BSA/1x Steinberg's solution (Asashima *et al*., 1990) until sibling embryos reached stage 11, 25, or 36. Embryos were staged according to Nieuwkoop and Faber (1967).

Surface plasmon resonance studies

Binding experiments were performed using the BIACORE2000 surface plasmon resonance biosensor (Biacore AB, Uppsala). Purified rxBMP-4 was immobilized on the sensor chip surface (CM5, certified grade, Biacore AB) by the amine coupling method as described previously (Iemura *et al*., 1998). The immobilization level for BMP-4 was 2904 RU. For binding studies, hFSS or hFSL (5 μg/ml) was injected over the BMP-4 surface at a flow rate of 20 μl/min for 120 s at 25 °C. For the blank runs, both samples were injected over mock-coupled sensor chip surfaces containing no protein simultaneously with each experimental run. All curves were corrected for background by subtracting the blank run, using BIAevaluation software version 3.0 (BIACORE AB).

Western blot analysis and immunofluorescence

A Flag (DYKDDDDK) or HA (YPYDVPDYA) epitope was N-terminally inserted between Leu34 and Gln35 of the xFSS and xFSL sequences. The synthetic oligonucleotides used were

top, 5'-GGATTACAAGGATGACGATGACAAGCTGCA-3'; bottom, 5'-GCTTGTCATCGTCATCCTTGTAATCCTGCA-3' for the Flag and top, 5'-GTATCCATATGATGTGCCAGATTATGCACTGCA-3'; bottom, 5'-GTGCATAATCTGGCACATCATATGGATACTGCA-3' for the HA epitope. The top and bottom oligonucleotides were annealed and then ligated into linearized (digested with *Pst*I) xFSS and xFSL constructs.

293T cells were transiently transfected with the Flag-xFSS and Flag-xFSL constructs using the calcium phosphate method (Chen and Okayama, 1987). Twenty-four hours after transfection the culture medium was replaced with serum-free medium and after an additional twenty-four hours the conditioned medium and cells were collected. The 293T cell culture fractions and animal caps were subjected to Western blot analysis as described in Iemura *et al* (1998). To compare the localization xFSS and xFSL proteins, immunofluorescence analysis of the dissected ectodermal explants of *Xenopus* embryos injected with Flag- or HA-tagged xFSS and xFSL, was performed as described previously (Larabell *et al*., 1997).

The anti-Flag antibody M2 and polyclonal anti-HA antibody were purchased from SIGMA and Santa Cruz Biotech, respectively.

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References

ASASHIMA, M., NAKANO, H., SHIMADA, K., KINOSHITA, K., ISHII, K., SHIBAI, H., and UENO, N. (1990). Mesoderm induction in early amphibian embryos by activin A (erythroid differentiation factor). *Roux's Arch. Dev. Biol.* 198: 330-335.

ASHE, H. L., and LEVINE, M. (1999). Local inhibition and long-range enhancement of Dpp signal transduction by Sog. *Nature* 398: 427-431.

BOUWMEESTER, T., KIM, S., SASAI, Y., LU, B., and DE ROBERTIS, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* 382: 595-601.

CHEN, C., and OKAYAMA, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7: 2745-2752.

DOSCH, R., GAWANTKA, V., DELIUS, H., BLUMENSTOCK, C., and NIEHRS, C. (1997). Bmp-4 acts as a morphogen in dorsoventral mesoderm patterning in Xenopus. *Development* 124: 2325-2334.

FAINSOD, A., DEISSLER, K., YELIN, R., MAROM, K., EPSTEIN, M., PILLEMER, G., STEINBEISSER, H., and BLUM, M. (1997). The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech. Dev.* 63: 39-50.

GRAFF, J. M. (1997). Embryonic patterning: to BMP or not to BMP, that is the question. *Cell* 89: 171-174.

GREEN, J. B., NEW, H. V., and SMITH, J. C. (1992). Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* 71: 731-739.

HEMMATI-BRIVANLOU, A., and MELTON, D. (1997). Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell* 88: 13-17.

HEMMATI-BRIVANLOU, A., KELLY, O. G., and MELTON, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* 77: 283-295.

HOLLEY, S. A., JACKSON, P. D., SASAI, Y., LU, B., DE ROBERTIS, E. M., HOFFMANN, F. M., and FERGUSON, E. L. (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. *Nature* 376: 249-253.

HOLLEY, S. A., NEUL, J. L., ATTISANO, L., WRANA, J. L., SASAI, Y., O'CONNOR, M. B., DE ROBERTIS, E. M., and FERGUSON, E. L. (1996). The Xenopus dorsalizing factor noggin ventralizes Drosophila embryos by preventing DPP from activating its receptor. *Cell* 86: 607-617.

HSU, D. R., ECONOMIDES, A. N., WANG, X., EIMON, P. M., and HARLAND, R. M. (1988). The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Molecular Cell* 1: 673-683.

IEMURA, S., YAMAMOTO, T. S., TAKAGI, C., UCHIYAMA, H., NATSUME, T., SHIMASAKI, S., SUGINO, H., and UENO, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* 95: 9337-9342.

KISHIMOTO, Y., LEE, K. H., ZON, L., HAMMERSCHMIDT, M., and SCHULTE-

MERKER, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* 124: 4457-4466.

LARABELL, C. A., TORRES, M., ROWNING, B. A., YOST, C., MILLER, J. R., WU, M., KIMELMAN, D., and MOON, R. T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* 136: 1123-1136.

LEYNS, L., BOUWMEESTER, T., KIM, S. H., PICCOLO, S., and DE ROBERTIS, E. M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* 88: 747-756.

MILLER-BERTOGLIO, V. E., FISHER, S., SANCHEZ, A., MULLINS, M. C., and HALPERN, M. E. (1997). Differential regulation of chordin expression domains in mutant zebrafish. *Dev. Biol.* 192: 537-550.

MIYAZONO, K., HELLMAN, U., WERNSTEDT, C., and HELDIN, C. H. (1988). Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *J. Biol. Chem.* 263: 6407-6415.

MOON, R. T., BROWN, J. D., YANG-SNYDER, J. A., and MILLER, J. R. (1997). Structurally related receptors and antagonists compete for secreted Wnt ligands. *Cell* 88: 725-728.

NAKAMURA, T., TAKIO, K., ETO, Y., SHIBAI, H., TITANI, K., and SUGINO, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* 247: 836-838.

NIEUWKOOP, P. D., and FABER, J. (1967). A normal table of Xenopus laevis. North Holland Publishing Co., Amsterdam.

NIKAIDO, M., TADA, M., SAJI, T., and UENO, N. (1997). Conservation of BMP signaling in zebrafish mesoderm patterning. *Mech. Dev.* 61: 75-88.

NIKAIDO, M., TADA, M., TAKEDA, H., KUROIWA, A., and UENO, N. (1999). In vivo analysis using variants of zebrafish BMPR-IA: range of action and involvement of BMP in ectoderm patterning. *Development* 126: 181-190.

PERRIMON, N., and MCMAHON, A. P. (1999). Negative feedback mechanisms and their roles during pattern formation. *Cell* 97: 13-16.

PICCOLO, S., AGIUS, E., LEYNS, L., BHATTACHARYYA, S., GRUNZ, H., BOUWMEESTER, T., and DE ROBERTIS, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* 397: 707-710.

PICCOLO, S., SASAI, Y., LU, B., and DE ROBERTIS, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* 86: 589-598.

SALIC, A. N., KROLL, K. L., EVANS, L. M., and KIRSCHNER, M. W. (1997). Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of Xenopus embryos. *Development* 124: 4739-4748.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L. K., and DE ROBERTIS, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer- specific homeobox genes. *Cell* 79: 779-790.

SHIMASAKI, S., KOGA, M., ESCH, F., COOKSEY, K., MERCADO, M., KOBA, A., UENO, N., YING, S. Y., LING, N., and GUILLEMIN, R. (1988). Primary structure of the human follistatin precursor and its genomic organization. *Proc. Natl. Acad. Sci. USA* 85: 4218-4222.

SMITH, W. C., and HARLAND, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* 70: 829-840.

SUGINO, K., KUROSAWA, N., NAKAMURA, T., TAKIO, K., SHIMASAKI, S., LING, N., TITANI, K., and SUGINO, H. (1993). Molecular heterogeneity of follistatin, an activin-binding protein. Higher affinity of the carboxyl-terminal truncated forms for heparan sulfate proteoglycans on the ovarian granulosa cell. *J. Biol. Chem.* 268: 15579-15587.

SUZUKI, A., KANEKO, E., MAEDA, J., and UENO, N. (1997). Mesoderm induction by BMP-4 and -7 heterodimers. *Biochem. Biophys. Res. Commun.* 232: 153-156.

SUZUKI, A., SHIODA, N., and UENO, N. (1995). Bone morphogenetic protein acts as a ventral mesoderm modifier in early Xenopus embryos. *Dev. Growth Differ.* 37: 581-588.

TASHIRO, K., YAMADA, R., ASANO, M., HASHIMOTO, M., MURAMATSU, M., and SHIOKAWA, K. (1991). Expression of mRNA for activin-binding protein (follistatin) during early embryonic development of *Xenopus laevis. Biochem. Biophys. Res. Commun.* 174: 1022-1027.

THOMSEN, G. H. (1997). Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning. *Trends Genet.* 13: 209-211.

UENO, N., LING, N., YING, S. Y., ESCH, F., SHIMASAKI, S., and GUILLEMIN, R. (1987). Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle- stimulating hormone. *Proc. Natl. Acad. Sci. USA* 84: 8282-8286.

WANG, S., KRINKS, M., LIN, K., LUYTEN, F. P., and MOOS, M., JR. (1997). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* 88: 757-766.

WILSON, P. A., and HEMMATI-BRIVANLOU, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* 376: 331-333.

YAMAMOTO, T. S., TAKAGI, C., and UENO, N. (2000). Requirement of Xmsx-1 in the BMP-triggered ventralization of Xenopus embryos. *Mech. Dev.* 91: 131-141.

ZIMMERMAN, L. B., DE JESUS-ESCOBAR, J. M., and HARLAND, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86: 599-606.

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